# Differential effects of α1-acid glycoprotein on bovine neutrophil respiratory burst activity and IL-8 production

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## ABSTRACT

During bacterial-mediated diseases, neutrophils (PMNs) play a critical role in defending the host against invading pathogens. PMN production of reactive oxygen species (ROS) contributes to the bactericidal capabilities of these cells. ROS are produced intracellularly and can be released extracellularly. The aberrant extracellular release of ROS, however, has been reported to induce injury to host tissues during mastitis and other inflammatory-mediated diseases of cattle. The acute phase response, which occurs shortly after infection or tissue injury, is characterized by the induction of a large number of plasma proteins referred to as acute phase proteins (APP). α₁-Acid glycoprotein (AGP) is an APP that increases in response to infection or injury in cattle and humans. The precise function of AGP is unknown, but it has been reported to possess anti-inflammatory properties. The objective of this study was to evaluate the effects of bovine AGP on PMN pro-inflammatory responses, including respiratory burst activity and cytokine production. Bovine AGP dose-dependently inhibited zymosan-induced PMN extracellular release of superoxide anion and hydrogen peroxide without affecting the capacity of PMN to engulf and kill *Staphylococcus aureus*. Moreover, AGP exerted its effect on ROS production regardless of whether PMNs were exposed to AGP prior to or after activation. In contrast to respiratory burst activity, AGP enhanced PMN production of IL-8. The precise mechanism by which AGP regulates PMN functions remains unknown, but data presented in this study suggest that AGP may have a complex role by differentially regulating PMN pro-inflammatory activities.

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## 1. Introduction

In response to bacterial infection, neutrophils (PMNs) are rapidly recruited to defend the host against the invading pathogen. Several chemoattractants are known to be involved in PMN recruitment, including the cytokine interleukin (IL)-8 (Persson et al., 1993; Harada et al., 1994). Activated PMNs, themselves, can release IL-8 (Cassatella, 1995), which has been shown to stimulate PMN adherence, degranulation, and reactive oxygen species production (ROS) (Peveri et al., 1988). The production of ROS, which is also referred to as respiratory or oxidative burst activity, is critical for host defense as impaired PMN oxidative burst

activity is associated with increased susceptibility to infection (Baehner, 1990; Dinauer, 1993).

ROS production is mediated by NADPH oxidase, a membrane associated multi-component protein enzyme complex that is assembled in an active form upon PMN stimulation (Hampton et al., 1998; Babior, 2000). ROS are detected both intracellularly and extracellularly (Hampton et al., 1998), the latter of which has been attributed to ROS escape from incompletely sealed pseudopods during phagocytosis (Babior, 1984; Dahlgren and Karlsson, 1999). In diseases such as mastitis, where PMN concentrations can approach 50 million cells/mL of milk (Bannerman et al., 2004), aberrant release of extracellular ROS can induce direct injury to host tissue, including the mammary epithelium (Capuco et al., 1986; Ledbetter et al., 2001; Lauzon et al., 2005).

Shortly after tissue injury caused by trauma, inflammation or infection, an early non-specific defense mechanism, known as the acute phase response (APR), is elicited by the host to both enhance the host innate immune response to infection and limit the potential harmful effects of the inflammatory response on healthy tissues (Suffredini et al., 1999). The APR is characterized by systemic inflammatory signs including fever and increases in concentrations of a large number of plasma proteins called acute phase proteins (APP). $\alpha_1$-Acid glycoprotein (AGP) is an APP with anti-inflammatory properties that is expressed in humans and cattle (Van Dijk and Poland, 2003; Ceciliani et al., 2007). AGP serum concentrations in healthy Holstein cows are ~0.3 mg/mL (Tamura et al., 1989) and these concentrations increase 2- to 4-fold in response to systemic infection or injury (Eckersall et al., 2001). The precise nature of AGP function is still unknown; however, in several studies it has been shown to act as a serum binding protein and to modulate the host inflammatory response (Logdberg and Wester, 2000). In mice challenged with TNF or LPS, AGP pre-treatment has been shown to improve survival (Libert et al., 1994). *In vitro*, AGP has been shown to induce macrophages expression of IL-1 receptor antagonist and soluble TNF receptor, which antagonize the activity of IL-1β and TNF-α, respectively (Tilg et al., 1993; Hochepied et al., 2003). In contrast to these anti-inflammatory properties, AGP has been reported to potentiate LPS-induced monocyte secretion of pro-inflammatory cytokines (Boutten et al., 1992).

A number of studies have also investigated the effects of AGP on PMN functions. At physiological concentrations, human AGP has inhibitory effects on PMN chemotactic responses after stimulation with formyl-methionyl-leucyl-phenylalanine and the complement component C5a (Laine et al., 1990; Vasson et al., 1994). Moreover, it has been shown that low doses of AGP promote PMN aggregation, while higher doses inhibit this response (Laine et al., 1990). PMN respiratory burst activity is also reported to be modulated by AGP, and previous studies have demonstrated that human AGP can inhibit PMN extracellular release of superoxide anion after activation with opsonized zymosan or phorbol 12-myristate,13-acetate (PMA) (Costello et al., 1984; Vasson et al., 1994). Much less is known about the function of bovine AGP in moderating respiratory burst activity. The objectives of this study were to evaluate the effects of bovine AGP on PMN respiratory burst activity and to elucidate whether AGP globally modulates other PMN functions, including cytokine production, phagocytosis, and bacterial killing.

## 2. Materials and methods

### 2.1. Reagents

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and isoluminol (6-amino-2,3-dihydro-1,4-phthalazinedione) (both from Sigma Chemical Co., St. Louis, MO) were prepared as a 1-M stock solution in dimethyl sulfoxide (DMSO). PMA (Sigma Chemical Co.), and diphenyleneiodonium chloride (DPI) (Calbiochem-Novabiochem Corp., San Diego, CA) were prepared as 10 mM stock solutions in DMSO. Cytochrome *c* from bovine heart and superoxide dismutase (SOD) from bovine erythrocytes (both from Sigma Chemical Co.) were prepared as 1 mM and 1000 U/mL stock solutions, respectively, in calcium- and magnesium-free Hanks' balanced salt solution (CMF-HBSS). Catalase, isolated from bovine liver, was obtained as a prepared suspension in water (Sigma Chemical Co.). Peroxidase type VI from horseradish (HRP) (Sigma Chemical Co.) was prepared as a stock solution of 500 U/mL in endotoxin-free water. Bovine serum albumin fraction V solution (BSA) (Sigma Chemical Co.) was prepared as a 20-mg/mL stock solution in phenol red-free RPMI media (Cambrex BioScience, Inc., Walkersville, MD), supplemented with 20 mM HEPES (Sigma Chemical Co.) and 10% heat inactivated fetal bovine serum (FBS) (Invitrogen Corp., Grand Island, NY).

Zymosan was activated by suspending 500 mg of zymosan A from *Saccharomyces cerevisiae* (Sigma Chemical Co.) in 50 mL of HBSS. The suspension was boiled for 20 min, mixed in a blender for 30 s, and then centrifuged at $250 \times g$ for 10 min. The pellet was washed twice by resuspension in HBSS and recentrifugated as above. After the final wash, zymosan was resuspended in 50 mL of a 65% solution of pooled bovine serum diluted in phosphate-buffered saline (PBS). Following a 1-h incubation at 37 °C, the suspension was centrifuged and the resulting pellet washed twice as above. After the final wash, activated zymosan was resuspended in HBSS to a final concentration of 10 mg/mL, aliquotted, and stored at −70 °C.

AGP was purified from bovine serum as previously described (Ceciliani et al., 2007). The endotoxin content of the purified bovine AGP, as assessed with the Limulus amoebocyte lysate assay (Cambrex BioScience, Inc.), was <0.05 ng/mg of protein. After purification, AGP was resuspended to a final concentration of 20 mg/mL in phenol red-free RPMI media, supplemented with 20 mM HEPES, and 10% heat inactivated FBS.

### 2.2. Cows

Clinically healthy lactating Holstein cows from the USDA-ARS Beltsville dairy herd were used as blood donors for all experiments. The use and care of all animals in this study were approved by the Beltsville Area Animal Care and Use Committee.

### 2.3. Isolation of bovine blood PMNs

Blood was obtained from the coccygeal vein and collected into Vacutainer® glass tubes containing acid–citrate–dextrose (Becton Dickinson Corp., Franklin, Lakes, NJ), inverted 5×, and stored on ice. PMNs were isolated using a Percoll gradient as previously described (Rinaldi et al., 2007). The purity of the isolated PMNs was >95% and viability >90%. PMN concentrations were adjusted with CMF-HBSS, PBS or media, and maintained on ice until used in the various assays. All assays for the measurement of PMN ROS production (briefly described below) have been previously validated (Rinaldi et al., 2007).

### 2.4. Luminol chemiluminescence (CL) assay

$2 \times 10^5$ PMNs were suspended in 100 µl of CMF-HBSS in the wells of a 96-well plate and incubated for 10 min at 37 °C with 16 µl of media or varying concentrations of either AGP or BSA. HBSS was used as the diluent to adjust the concentrations of the various solutions. Twenty microliter of luminol (5 mM) and 60 µl of HBSS, zymosan (833 µg/mL) or PMA (133 nM) were then added, and the final volume of each well adjusted to 200 µl with HBSS. For evaluation of the effects of pre- and post-activation exposure to AGP on ROS production, respectively, the reactions were set up as above except that 16 µl of media, DPI (100 µM), AGP (2.5 mg/mL) or BSA (2.5 mg/mL) were added to PMNs 10 min prior to or 45 min after the addition of luminol and either HBSS, zymosan or PMA. Immediately following PMN activation, CL was measured every 5 min with a Veritas microplate luminometer (Turner Biosystems, Inc., Sunnyvale, CA). Background values, defined as the mean CL values of luminol diluted in HBSS, were subtracted from all readings.

### 2.5. Isoluminol CL assay

Reactions with isoluminol were set up and monitored exactly as described for those with luminol except that luminol was substituted with 20 µl of isoluminol (500 µM) and 5 µl of HRP (160 U/mL), and 2 µl of either DPI (800 µM) or SOD (1000 U/mL), or 16 µl of AGP (5 mg/mL) or BSA (5 mg/mL), were added to wells as described for the luminol CL assay.

### 2.6. Cytochrome c reduction assay

$2 \times 10^5$ PMNs were suspended in 100 µl of CMF-HBSS in the wells of a 96-well plate and incubated for 10 min at 37 °C with 16 µl of media or increasing concentrations of either AGP or BSA. Ten microliters of cytochrome c (1 mM) and 50 µl of HBSS or zymosan (1 mg/mL) were then added, and the final volume of all wells adjusted to 200 µl with HBSS. For evaluation of the effects of pre- and post-activation exposure to AGP on ROS production, respectively, the reactions were set up as above except that 2 µl of either DPI (800 µM) or SOD (1000 U/mL), or 16 µl of media, AGP (5 mg/mL) or BSA (5 mg/mL), were added 10 min prior to or 30 min after the addition of cytochrome c and HBSS or zymosan. Immediately following PMN

activation, absorbance was measured on a plate reader (Bio-Tec Instruments, Inc., Winooski, VT). Optical density (OD) was measured at 10 min intervals at a wavelength of 550 nm and a reference wavelength of 670 nm, and the difference of the two ODs recorded. Background values, calculated from wells with cytochrome c diluted in HBSS, were subtracted from all values.

### 2.7. Amplex red fluorescence assay

The Amplex red-dependent fluorescence assay was performed according to the manufacturer's instructions (Molecular Probes, Inc.). $1.5 \times 10^4$ PMNs were suspended in 10 µl of Krebs Ringer phosphate glucose solution (KRPG solution; 145 mM NaCl, 5.7 mM $Na_2HPO_4$, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$ 5.5 mM glucose) in the wells of a 96-well plate and incubated for 10 min at 37 °C with 9.6 µl of media or increasing concentrations of either AGP or BSA. Eighty microliter of Amplex red reaction mixture [75 µl of Amplex red (10 mM) and 150 µl of HRP (10 U/mL) diluted in 11.8 mL of KRPG solution] and 20 µl of HBSS or zymosan (1.5 mg/mL) were then added, and the final volume of all wells adjusted to 120 µl with HBSS. For evaluation of the effects of pre- and post-activation exposure to AGP on ROS production, respectively, the reactions were set up as above except that 1.2 µl of either DPI (800 µM) or catalase (100,000 U/mL), or 9.6 µl of media, AGP (7.5 mg/mL) or BSA (7.5 mg/mL), were added 10 min prior to or 30 min after the addition of Amplex red reaction mixture and HBSS or zymosan. Immediately following PMN activation, fluorescence was measured every 15 min with a plate reader (Bio-Tec Instruments, Inc.) at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Background values, calculated from the wells with the Amplex red reaction mixture diluted in HBSS, were subtracted from all readings.

### 2.8. PMN phagocytosis and killing

*Staphylococcus aureus* strain 305 (American Type Culture Collection, Manassas, VA) was cultured as previously described (Rinaldi et al., 2006). The stock culture was diluted in PBS to yield a final concentration of $1 \times 10^8$ cfu/mL. To assess PMN phagocytosis and killing, 200 µl of *S. aureus* ($2 \times 10^7$ cfu) were added to tubes with or without $2 \times 10^6$ PMNs suspended in 1 mL of PBS, 400 µl of pooled (n = 5 cows) heat-inactivated bovine serum, and 160 µl of AGP (10 mg/mL), BSA (10 mg/mL), DPI (100 µM), or media. The reaction volume was adjusted to 2 mL with PBS and the samples were placed on an orbital shaker for 60 min at 39 °C. All reactions were set up in duplicate. To determine the percentage of PMN containing phagocytosed bacteria and the actual number of phagocytosed bacteria per PMN, Wright-stained cytospin centrifuge slides were prepared using a 50-µl aliquot of each reaction (Dulin et al., 1982). The first 100 PMN encountered in the field of view of a light microscope were scored as either positive or negative for intracellular bacteria. For those PMN scored as positive, the number of intracellular bacteria was enumerated. To evaluate whether AGP affected the bactericidal activity of PMN, 1.9 mL of the

remaining reaction were sonicated with a Virsonic ultrasonic cell disrupter 100 (Virtis Co., Gardiner, NY) at a power setting of 6 for 30 s. Rupture of the PMN was verified by microscopic examination. Sonication had no effect on bacterial viability (data not shown). A 0.1-mL aliquot from each sonicated reaction was serially diluted in PBS and 0.1 mL quantities of the resulting dilutions were spread on blood agar plates. The plates were incubated for 18 h at 37 °C and the colonies enumerated. The percentage of bacteria killed was determined by calculating the difference in the number of bacteria incubated in the absence and presence of PMN and dividing this difference by the number of bacteria incubated in the absence of PMN.

### 2.9. IL-8 ELISA

$6 \times 10^5$ PMNs suspended in 100 µl of complete medium (RPMI supplemented with 5% heat inactivated FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine) were incubated for 10 min at 37 °C with 16 µl of media, increasing concentration of AGP, or BSA (10 mg/mL), or 2 µl of DPI (800 µM). Fifty microliter of HBSS, zymosan (1 mg/mL), or PMA (160 nM) were then added, and the final volume of all wells adjusted to 200 µl with HBSS. After a 24-h incubation at 37 °C, plates were centrifuged ($1000 \times g$) for 5 min, the supernatants were collected, and stored at −30 °C. Supernatant IL-8 concentrations were determined with a commercially available human IL-8 ELISA kit (R&D Systems, Inc., Minneapolis, MN). The antibody pairs used in this kit have been previously shown to cross-react with bovine IL-8 (Shuster et al., 1996, 1997). The optical density at 450 nm and a correction wavelength of 565 nm were measured on a microplate reader. Values expressed in picograms per milliliter were extrapolated from a standard curve of known amounts of human IL-8 using linear regression.

### 2.10. Statistical methods

Absorbance, chemiluminescence, and fluorescence were continuously measured for all ROS assays at regular intervals up to 165 min post-stimulation. To enable calculation of cumulative ROS production, the area under the curve was calculated from plotted data points for each experimental condition using GraphPad Prism Version 4.00 for Windows (GraphPad Software, Inc., San Diego, CA). A one-way analysis of variance (ANOVA) (GraphPad Software, Inc.) in combination with the Dunnett *post hoc* comparison test was used to compare the mean responses between experimental groups when the data followed a Gaussian (normal) distribution. The Kruskal–Wallis nonparametric test (GraphPad Instat Software, Inc.) was used to compare the mean responses between experimental groups when the data did not follow a Gaussian (normal) distribution. Data generated from the luminol-dependent CL assay were $\log_{10}$ transformed prior to analyses. Statistical analyses were performed on raw data for all other assays. A *P*-value of <0.05 was considered significant.

## 3. Results

### 3.1. AGP inhibits bovine PMN respiratory burst activity as measured by luminol-dependent CL

Luminol-derived CL, which is elicited in response to the generation of an array of intracellular and extracellular ROS (Saez et al., 2000; Munzel et al., 2002; Rinaldi et al., 2007), was used to assess the effect of AGP on zymosan-induced PMN respiratory burst activity. AGP dose-dependently inhibited the luminol-dependent CL evoked by zymosan-stimulated PMNs (Fig. 1A). At a concentration as low as 200 µg/mL, AGP inhibited ∼65% of the evoked CL, whereas at concentrations ≥400 µg/mL it inhibited >90% of this response. BSA, which was evaluated in parallel as a control, failed to show any inhibitory effects (Fig. 1B). In other studies, the effects of pre- and post-activation exposure to AGP on PMN respiratory burst activity were evaluated. Exposure to a fixed concentration of AGP (200 µg/mL) blocked PMN-evoked luminol-dependent CL when added 10 min prior to (Fig. 1C) or 45 min after (Fig. 1D) zymosan-induced activation. Regardless of whether PMNs were exposed to AGP prior to or after stimulation, AGP was able to inhibit ∼75% of the evoked CL. DPI, which is an inhibitor of NADPH oxidase, blocked >95% of the PMN evoked CL regardless of whether it was added prior to or after PMN activation. The negative control, BSA (200 µg/mL), demonstrated no inhibitory effects regardless of the sequence of exposure.

To determine whether AGP's effect on PMNs was stimulus-dependent, the effect of AGP on PMA-activated PMNs was also assessed. Similar to its effect on PMNs stimulated with zymosan, AGP was able to dose-dependently inhibit luminol-dependent CL of PMA-activated PMNs (Fig. 2A), whereas BSA had no effect (Fig. 2B). At concentrations as low as 200 µg/mL, AGP inhibited ∼62% of the evoked CL, whereas at concentrations ≥400 µg/mL it inhibited >90% of this response. In studies evaluating the effects of pre- and post-activation exposure, AGP (200 µg/mL) inhibited the evoked CL regardless of whether PMN's were exposed to AGP 10 min prior to or 45 min after activation with PMA (Fig. 2C and D). The NADPH oxidase inhibitor DPI inhibited >90% of this response regardless of the sequence of exposure, whereas BSA had no inhibitory effect.

### 3.2. AGP inhibits bovine PMN generation of extracellular superoxide anion

Isoluminol-dependent CL, which is a specific indicator of the generation of extracellular superoxide anion (Lundqvist and Dahlgren, 1996; Dahlgren and Karlsson, 1999; Rinaldi et al., 2007), was used to assess whether AGP could inhibit PMN generation of this particular ROS. AGP dose-dependently inhibited isoluminol-dependent CL evoked by zymosan-stimulated PMNs (Fig. 3A). At a concentration as low as 200 µg/mL, AGP inhibited ∼46% of the evoked CL, whereas at concentrations ≥400 µg/mL it inhibited ≥90% of this response. In contrast, BSA did not demonstrate any inhibitory effects (Fig. 3B). In studies evaluating the effect of pre- and post-activation exposure
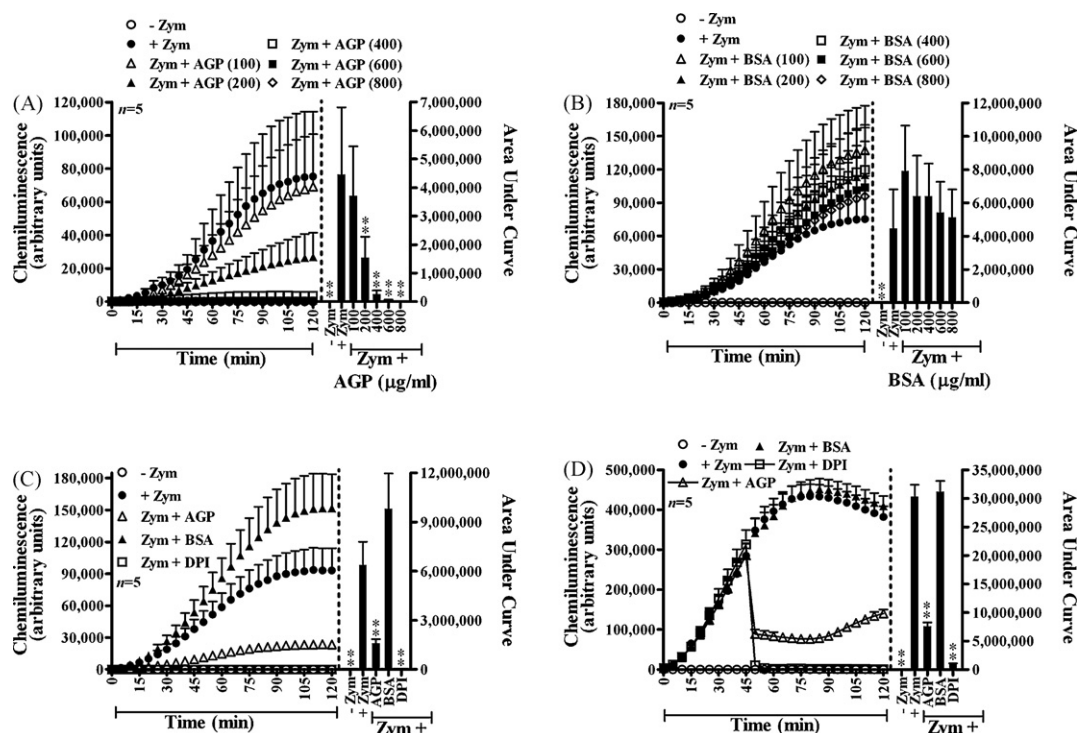
**Fig. 1.** Effect of AGP on zymosan-activated bovine neutrophil (PMN) oxidative burst activity measured by luminol-dependent chemiluminescence. Blood PMNs isolated from cows were exposed to increasing concentrations of AGP (A) or BSA (B) for 10 min. PMNs were subsequently stimulated with opsonized zymosan (Zym; 250 μg/mL) in the presence of luminol, and chemiluminescence measured every 5 min (left y-axis). In other studies, luminol-dependent chemiluminescence (left y-axis) was assayed in blood PMNs exposed to media, AGP (200 μg/mL), BSA (200 μg/mL), or diphenyleneiodonium chloride (DPI; 8 μM), 10 min prior to (C) or 45 min after (D) the addition of luminol and zymosan. For all studies, the area under the curve for each experimental condition was calculated (right y-axis). **Significantly decreased ($P < 0.01$) relative to PMNs stimulated with zymosan alone.

to AGP on PMN-evoked isoluminol dependent CL, exposure to a fixed concentration of AGP (400 μg/mL) blocked PMN-evoked isoluminol-dependent CL when added either 10 min prior to (Fig. 3C) or 45 min after (Fig. 3D) zymosan-induced activation. Regardless of whether PMNs were exposed to AGP prior to or after stimulation, AGP was able to exert its inhibitory effect. SOD, which scavenges extracellular superoxide, and DPI inhibited >55% and >85%, respectively, of the evoked CL regardless of whether they were added prior to or after PMN activation. BSA (400 μg/mL) failed to demonstrate any inhibitory effects regardless of the sequence of exposure.

To confirm the ability of AGP to specifically inhibit PMN generation of extracellular superoxide, zymosan-activated PMNs were exposed to increasing concentrations of AGP in the presence of cytochrome c, the reduction of which occurs specifically in response to the generation of extracellular superoxide anion (Munzel et al., 2002; Tarpey et al., 2004; Rinaldi et al., 2007). Similar to results obtained with the isoluminol assay, AGP dose-dependently inhibited extracellular superoxide anion production of zymosan-stimulated PMNs as assessed by cytochrome c reduction (Fig. 4A). At a concentration as low as 100 μg/mL, AGP inhibited ~34% of the measured cytochrome c reduction, whereas at concentrations ≥400 μg/mL it inhibited >65% of the response. BSA failed to show any inhibitory effects

(Fig. 4B). In studies evaluating the effect of pre- and post-activation exposure to AGP on PMN extracellular release of superoxide, and corresponding cytochrome c reduction, exposure to a fixed concentration of AGP (400 μg/mL) inhibited cytochrome c reduction when added 10 min prior to (Fig. 4C) or 30 min after (Fig. 4D) stimulation with zymosan. When PMNs were exposed to AGP prior to stimulation, AGP inhibited ~69% of cytochrome c reduction; however, when added 30 min after stimulation, AGP inhibited ~35% of the response. DPI inhibited ~60% and ~30% of the PMN extracellular release of superoxide when added prior to or after PMN activation, respectively. SOD inhibited >65% of the response regardless of whether it was added prior to or after PMN activation. BSA (400 μg/mL) demonstrated no inhibitory effects regardless of the sequence of exposure.

### 3.3. AGP inhibits bovine PMN generation of extracellular hydrogen peroxide

Amplex red-dependent fluorescence, which is specifically elicited in response to the generation of extracellular hydrogen peroxide (Zhou et al., 1997; Forteza et al., 2005; Rinaldi et al., 2007), was used to investigate the ability of AGP to inhibit extracellular generation of this particular ROS. AGP dose-dependently inhibited the Amplex red-
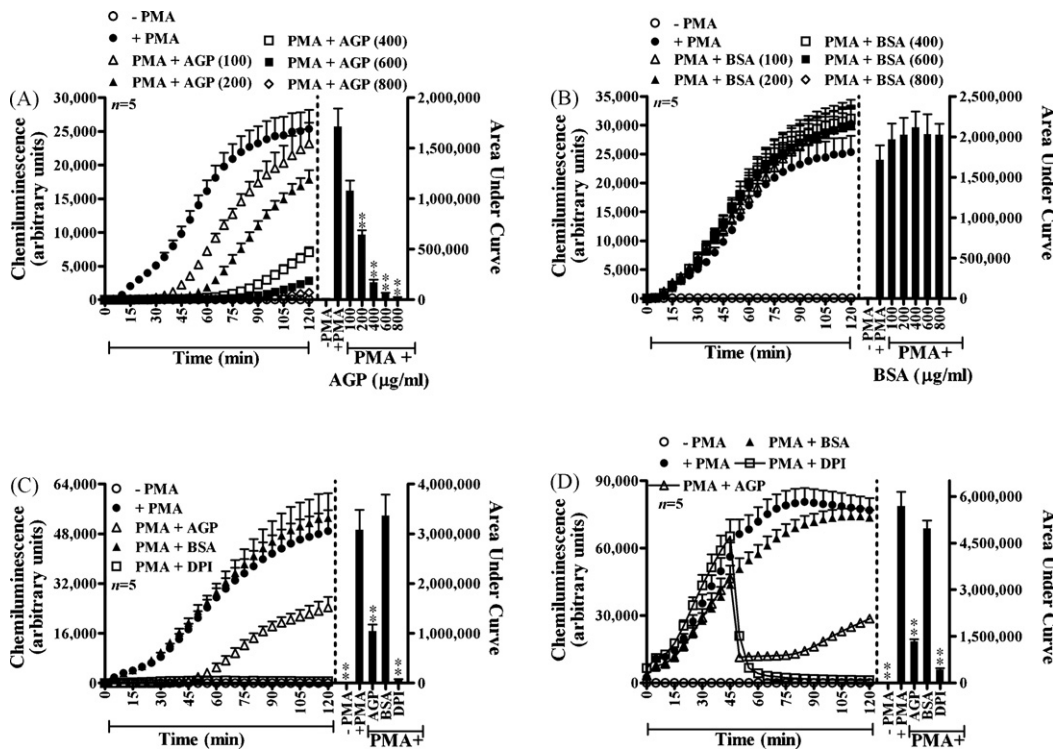
**Fig. 2.** Effect of AGP on PMA-activated bovine neutrophil (PMN) oxidative burst activity measured by luminol-dependent chemiluminescence. Blood PMNs isolated from cows were exposed to increasing concentrations of AGP (A) or BSA (B) for 10 min. PMNs were subsequently stimulated with PMA (40 nM) in the presence of luminol, and chemiluminescence measured every 5 min (left *y*-axis). In other studies, luminol-dependent chemiluminescence (left *y*-axis) was assayed in blood PMNs exposed to media, AGP (200 μg/mL), BSA (200 μg/mL), or DPI (8 μM), 10 min prior to (C) or 45 min after (D) the addition of luminol and PMA. For all studies, the area under the curve for each experimental condition was calculated (right *y*-axis). **Significantly decreased ($P < 0.01$) relative to PMNs stimulated with PMA alone.

dependent fluorescence evoked by zymosan-stimulated PMNs (Fig. 5A). At concentrations as low as 100 μg/mL, AGP inhibited ~45% of the evoked fluorescence, whereas at concentrations ≥600 μg/mL it completely abrogated this response. BSA failed to show any inhibitory effects (Fig. 5B). In studies evaluating the effect of pre- and post-activation exposure to AGP on PMN-evoked Amplex red fluorescence, exposure to a fixed concentration of AGP (600 μg/mL) blocked PMN-evoked fluorescence when added 10 min prior to (Fig. 5C) or 30 min after (Fig. 5D) zymosan-induced activation. When AGP was added 10 min prior to PMN stimulation, it completely abrogated the PMN extracellular release of hydrogen peroxide, whereas when it was added 30 min after stimulation, AGP inhibited >50% of the response. Catalase, which scavenges extracellular hydrogen peroxide, and DPI inhibited >60% of the PMN-evoked Amplex red fluorescence regardless of whether

they were added prior to or after PMN activation. BSA (600 μg/mL) demonstrated no inhibitory effects regardless of the sequence of exposure.

### 3.4. AGP does not affect PMN-mediated phagocytosis and killing of *S. aureus*

To determine whether the capacity of AGP to specifically inhibit the generation of extracellular ROS could affect PMN-mediated pathogen clearance, the effects of AGP on PMN phagocytosis and bactericidal activity were assessed (Table 1). PMNs were incubated with *S. aureus* for 60 min in the presence or absence of AGP (800 μg/mL), BSA (800 μg/mL) or DPI (8 μM). Regardless of exposure to media, AGP, BSA or DPI, equivalent percentages of PMNs contained phagocytosed bacteria. Further, the average number of phagocytosed bacteria within each PMN was

**Table 1**
Effect of AGP (800 μg/mL), BSA (800 μg/mL) and DPI (8 μM) on neutrophil (PMN) phagocytosis and killing of *Staphylococcus aureus*

|  | Media | AGP | BSA | DPI |
|---|---|---|---|---|
| PMN with phagocyted bacteria (%) | 76.7 ± 3.5 | 79.3 ± 3.4 | 77.5 ± 4.6 | 83.0 ± 4.3 |
| Number of phagocyted bacteria/PMN | 14.3 ± 1.4 | 15.1 ± 2.0 | 13.7 ± 1.5 | 14.1 ± 1.3 |
| Killing of bacteria (%) | 66.3 ± 3.3 | 67.1 ± 3.9 | 54.5 ± 4.3* | 40.2 ± 7.4** |

All data presented as mean ± standard error; *n* = 6.
*,**Significantly decreased ($P < 0.05$ and $P < 0.01$, respectively) relative to activated PMNs exposed to media alone.
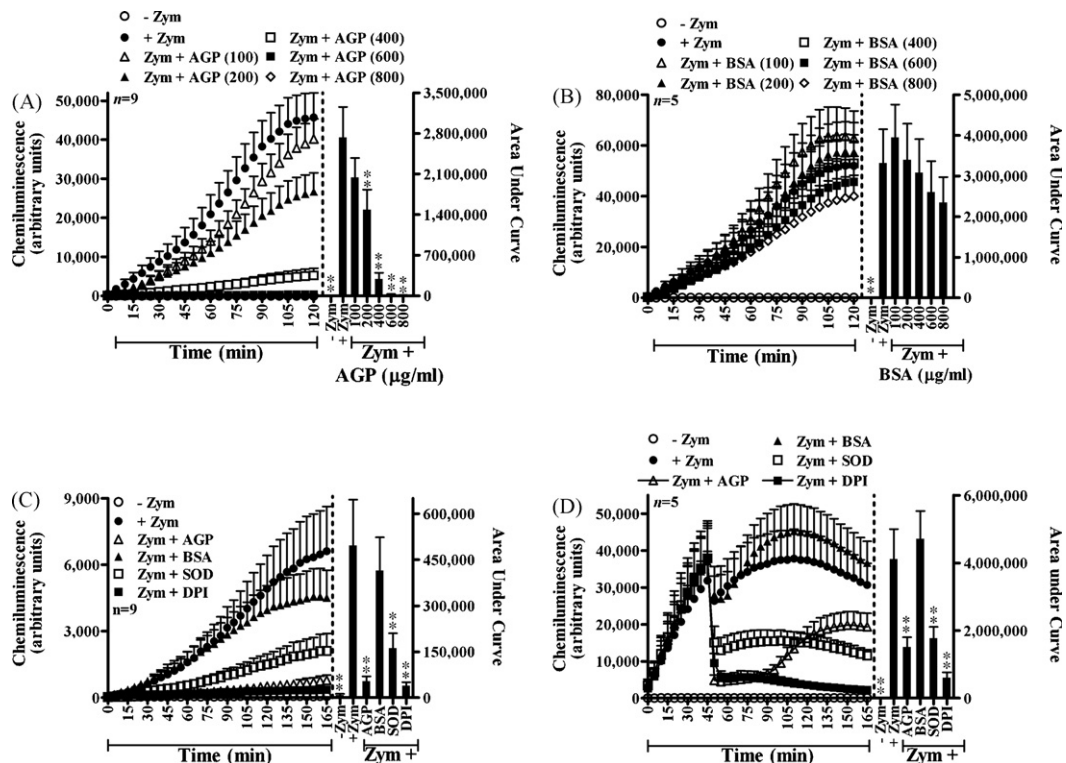
**Fig. 3.** Effect of AGP on bovine neutrophil (PMN) generation of extracellular superoxide anion as measured by isoluminol-dependent chemiluminescence. Blood PMNs isolated from cows were exposed to increasing concentrations of AGP (A) or BSA (B) for 10 min. PMNs were subsequently stimulated with opsonized zymosan (Zym; 250 μg/mL) in the presence of isoluminol, and chemiluminescence measured every 5 min (left y-axis). In other studies, isoluminol-dependent chemiluminescence (left y-axis) was assayed in blood PMNs exposed to media, AGP (400 μg/mL), BSA (400 μg/mL), superoxide dismutase (SOD; 10 U/mL) or DPI (8 μM), 10 min prior to (C) or 45 min after (D) the addition of isoluminol and zymosan. For all studies, the area under the curve for each experimental condition was calculated (right y-axis). **Significantly decreased ($P < 0.01$) relative to PMNs stimulated with zymosan alone.

equivalent irrespective of treatment. Exposure to AGP had no effect on PMN killing of *S. aureus*, whereas exposure to BSA or DPI impaired the capacity of PMNs to kill bacteria by ~18% and ~40%, respectively.

### 3.5. AGP enhances IL-8 production of bovine PMN

To determine whether AGP could interfere with other PMN functions, the influence of AGP on PMN production of IL-8 was assessed. Exposure of zymosan-activated PMNs to increasing concentrations of AGP caused a dose-dependent enhancement of IL-8 production (Fig. 6A). In the presence of 400 μg/mL or 800 μg/mL of AGP, activated PMNs showed an approximate 3- or 4.3-fold increase in IL-8 production, respectively, compared to activated PMN exposed to media. Similar to its effect on PMNs stimulated with zymosan, AGP also enhanced the IL-8 production of PMA-activated PMNs (Fig. 6B). Exposure to AGP (800 μg/mL) caused an increase in IL-8 production of ~1.74-fold. Regardless of the stimulus used to activate PMNs, exposure to the BSA (800 μg/mL) control had no effects on IL-8 production (Fig. 6 A and B).

### 4. Discussion

This study investigated the effects of bovine AGP on PMN respiratory burst activity. In healthy cows, AGP serum concentrations are ~300 μg/mL (Tamura et al., 1989), whereas in infected cows the concentrations are 2–4-fold higher (Eckersall et al., 2001). Thus, the concentrations evaluated in this study are comparable to those found under naturally occurring physiological and pathophysiological conditions. Further, the range of concentrations tested enabled the differentiation of AGP's effects on PMNs when present at levels commensurate with infection versus those present in healthy animals. At concentrations comparable to that of healthy animals (*i.e.*, 200 μg/mL), AGP inhibited between 46% and 65% of the PMN response in the various assays of ROS production. In contrast, a level of inhibition ranging from 64% to 100% of the PMN response was detected in the various ROS assays when PMNs were exposed to concentrations comparable to that of infected animals (*i.e.*, 800 μg/mL). These data suggest that AGP is able to partially impair PMN ROS production under physiological conditions, but that it exerts maximal inhibitory activity when present at levels associated with infection. Because ROS-mediated tissue damage has been reported to accompany PMN recruitment and activation (Capuco et al., 1986; Ledbetter et al., 2001; Lauzon et al., 2005), the ability of AGP to exert its protective effects at concentrations found during the course of infection may help in minimizing inflammation-associated tissue damage.

Assays that measure global ROS production or that selectively measure extracellular $O_2^-$ or $H_2O_2$ were used to
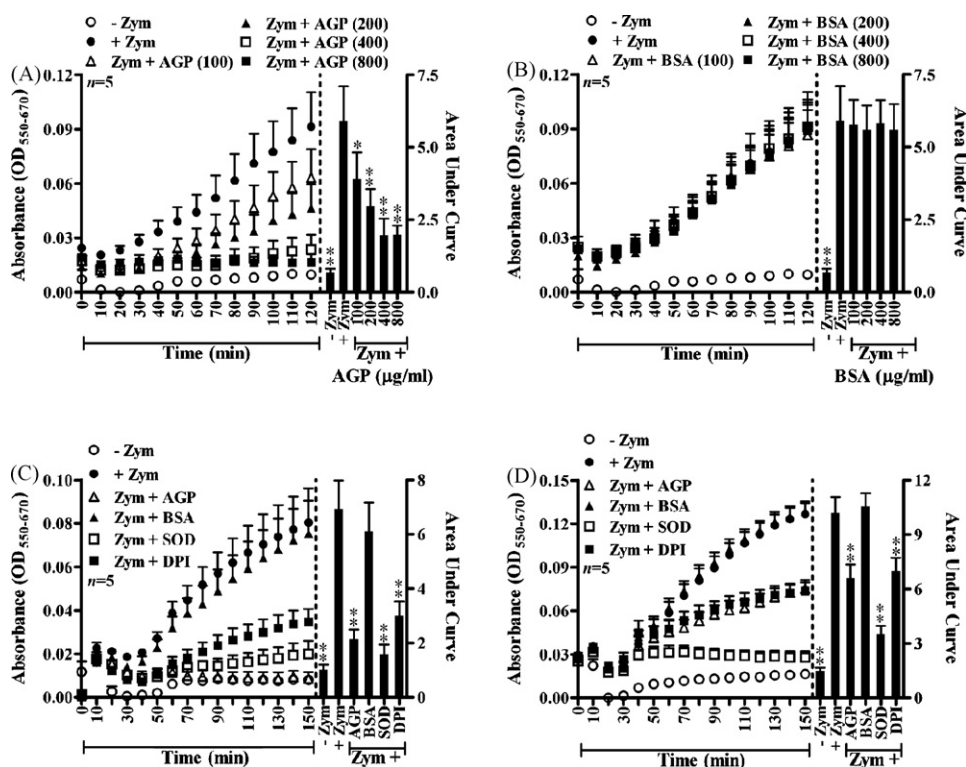
Fig. 4. Effect of AGP on bovine neutrophil (PMN) generation of extracellular superoxide as measured by cytochrome $c$ reduction. Blood PMNs isolated from cows were exposed to increasing concentrations of AGP (A) or BSA (B) for 10 min. PMNs were subsequently stimulated with opsonized zymosan (Zym; 250 $\mu$g/mL) in the presence of cytochrome $c$, and absorbance values measured every 10 min (left $y$-axis). In other studies, cytochrome $c$ reduction (left $y$-axis) was assayed in blood PMNs exposed to media, AGP (400 $\mu$g/mL), BSA (400 $\mu$g/mL), SOD (10 U/mL), or DPI (8 $\mu$M), 10 min prior to (C) or 30 min after (D) the addition of cytochrome $c$ and zymosan. For all studies, the area under the curve for each experimental condition was calculated (right $y$-axis). *,**Significantly decreased ($P < 0.05$ and $P < 0.01$, respectively) relative to PMNs exposed to zymosan alone.

evaluate the effects of bovine AGP on PMN respiratory burst activity. Luminol dependent CL, which is elicited in response to the generation of an array of intracellular and extracellular ROS (Rinaldi et al., 2007), has been commonly used as a comprehensive indicator of PMN respiratory burst activity. Because AGP inhibited luminol dependent CL (Figs. 1 and 2), more selective assays of ROS production were utilized to better understand if AGP's effects were selectively restricted to the generation of certain ROS. Using two distinct assays that specifically detect extracellular $O_2^-$ (i.e., isoluminol-dependent CL and cytochrome $c$ reduction), bovine AGP was established to inhibit the generation of this radical (Figs. 3 and 4). Previous studies investigating the effects of human AGP on PMN ROS production have solely used the cytochrome $c$ reduction assay to measure this response (Costello et al., 1984; Laine et al., 1990; Vasson et al., 1994), and consistent with our findings with bovine AGP, these studies demonstrated that human AGP inhibits the extracellular generation of $O_2^-$. Concentrations of human AGP ranging from 50 $\mu$g/mL to 500 $\mu$g/mL were shown to inhibit 20–50% of the cytochrome $c$ reduction evoked by zymosan-stimulated PMN (Laine et al., 1990). This level of inhibition was similar to that observed in this study when bovine PMNs were exposed to comparable concentrations of bovine AGP. Thus, human and bovine AGP exhibit similar down-

regulatory activity on PMN extracellular superoxide generation.

Extracellular $H_2O_2$ is rapidly formed in activated PMN from $O_2^-$ by spontaneous or enzymatic dismutation by superoxide dismutase (Weiss, 1989; Hampton et al., 1998). Thus, one may hypothesize that AGP's ability to inhibit PMN release of $O_2^-$ may also affect corresponding generation of extracellular $H_2O_2$. Consistent with this hypothesis, extracellular release of $H_2O_2$ was lower in PMNs exposed to AGP, as measured by Amplex red-dependent fluorescence (Fig. 5). To our knowledge, this is the first study to establish an inhibitory effect of AGP on PMN generation of extracellular $H_2O_2$.

A previous study has investigated the effects of AGP on bovine PMN respiratory burst activity in response to PMA and opsonized bacteria (Stakauskas et al., 2005). The authors demonstrated that AGP could inhibit generation of ROS production in PMA-activated PMN, whereas it had no effect on PMN ROS production evoked by exposure to opsonized bacteria. This study also investigated the effects of AGP on ROS production by bovine PMNs stimulated with PMA. Consistent with previous studies using luminol or luminol-derivatives (Rinaldi et al., 2007; Nakano et al., 1986), chemiluminescence generated in response to PMN activation with PMA (Fig. 2) was lower than that observed when PMNs were stimulated with zymosan (Fig. 1). The
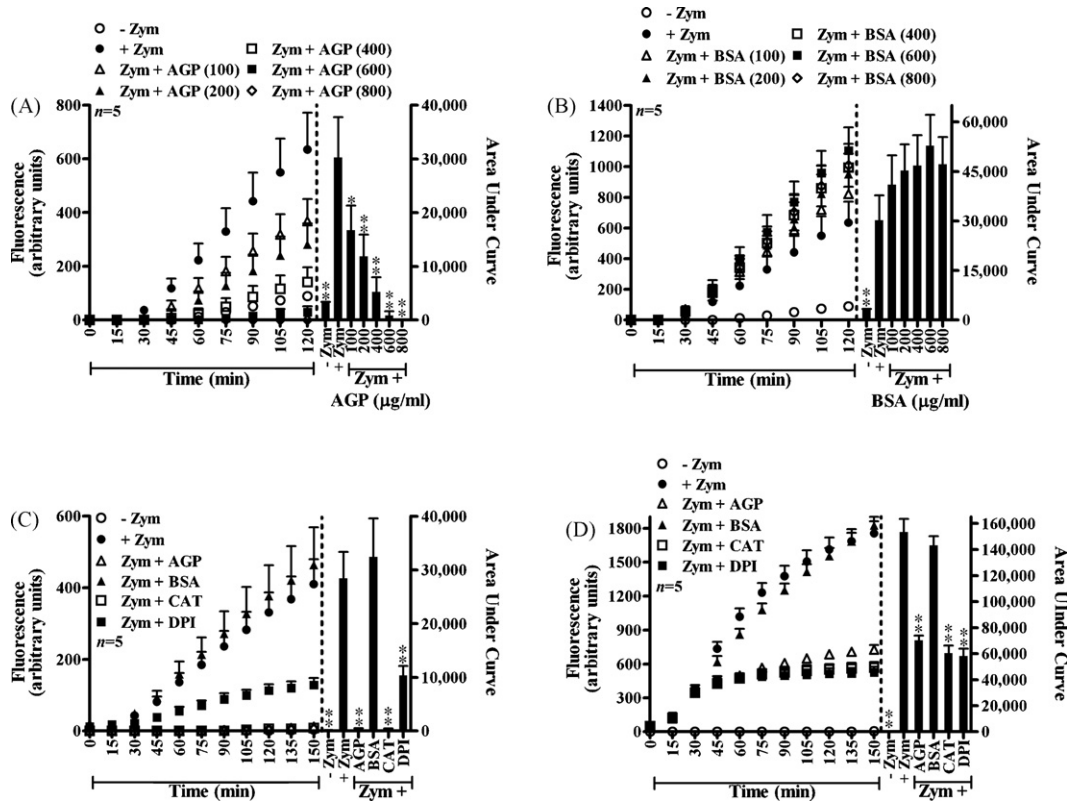
Fig. 5. Effect of AGP on bovine neutrophil (PMN) generation of extracellular hydrogen peroxide as measured by Amplex red-dependent fluorescence. Blood PMNs isolated from cows were exposed to increasing concentrations of AGP (A) or BSA (B) for 10 min. PMNs were subsequently stimulated with opsonized zymosan (Zym; 250 μg/mL) in the presence of Amplex red, and fluorescence values measured every 15 min (left y-axis). In other studies, Amplex red-dependent fluorescence (left y-axis) was assayed in blood PMNs exposed to media, AGP (600 μg/mL), BSA (600 μg/mL), catalase (CAT; 1000 U/mL), or DPI (8 μM), 10 min prior to (C) or 30 min after (D) the addition of Amplex red and zymosan. For all studies, the area under the curve for each experimental condition was calculated (right y-axis). *,**Significantly decreased ($P < 0.05$ and $P < 0.01$, respectively) relative to PMNs exposed to zymosan alone.

differential degree to which these agonists evoke luminol-dependent chemiluminescence may be due to the differential generation of specific ROS elicited by the two agonists and the corresponding differential sensitivity of the luminol assay to detect the individual ROS generated. Further, the differential nature by which PMA and zymosan activate may differentially affect the magnitude of the ROS response that is able to be evoked.

In contrast with the findings of Stakauskas et al. (2005), those reported here demonstrate that bovine AGP can inhibit PMN ROS production in response to PMA or opsonized zymosan and that the level of inhibition is comparable regardless of the stimulus used to activate PMNs (Figs. 1 and 2). Previous studies with human AGP have similarly demonstrated that AGP can inhibit PMN ROS production in response to PMA or opsonized zymosan
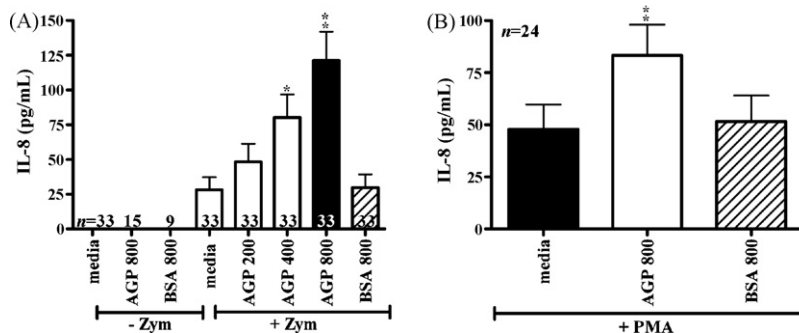


Fig. 6. Effect of AGP on bovine neutrophil (PMN) production of interleukin (IL)-8. Blood PMNs isolated from cows were exposed to increasing concentrations of AGP or a fixed concentration of BSA (800 μg/mL) for 10 min. PMNs were subsequently stimulated with opsonized zymosan (Zym; 250 μg/mL) (A) or PMA (40 nM) (B) and incubated for 24 h at 37 °C. Following stimulation, the IL-8 concentrations of the cell supernatants were determined by ELISA. *,**Significantly increased ($P < 0.05$ and $P < 0.01$, respectively) relative to zymosan-activated (A) or PMA-activated (B) PMNs exposed to media alone.

(Costello et al., 1984; Laine et al., 1990; Vasson et al., 1994). Thus, it is unclear why Stakauskas et al. (2005) identified an inhibitory role for AGP on ROS production of PMNs exposed to PMA, but not to opsonized bacteria. Interestingly, they concluded that AGP non-specifically binds PMA and prevents PMN activation. The data presented here with bovine AGP, and that presented previously with human AGP, contradicts this hypothesis, as AGP was shown to block activation by either PMA or opsonized zymosan.

To further discount the notion that AGP exerts its effect non-specifically by blocking stimulus activation of PMNs, we evaluated the effects of pre- and post-activation exposure to AGP on PMN ROS production. For those experiments evaluating the effect of post-activation exposure to AGP and the various inhibitors on ROS production, the optimal time (i.e., 30 min versus 45 min after activation) for introducing the inhibitors varied depending on the assay and the kinetics of the response as determined by the AGP dose–response studies. It is not surprising that the kinetics of the PMN response differed between experiments because the different experiments utilized different assays to measure specific ROS. In all assays of respiratory burst activity, AGP was able to inhibit PMN generation of ROS regardless of whether PMNs were exposed to AGP prior to or after activation with opsonized zymosan. A similar finding was also observed when PMA was used as the stimulus (Fig. 2). Finally, bovine AGP was shown to enhance, not inhibit, PMA and zymosan-induced IL-8 production (Fig. 6). Together, these findings preclude a mechanism of action by which AGP non-specifically blocks stimulus-induced activation of PMNs.

The actual mechanism by which AGP inhibits generation and/or release of ROS remains unknown. AGP may exert its effect directly on the NADPH oxidase complex, which drives ROS production. Alternatively, AGP may act as a scavenger of extracellular ROS. In an attempt to elucidate the mechanism of action, the effects of DPI, SOD, and catalase on PMN ROS production were evaluated in parallel with studies conducted with AGP. DPI, inhibits NADPH oxidase (Ellis et al., 1988; Hampton and Winterbourn, 1995), whereas SOD and catalase, scavenge extracellular $O_2^-$ and $H_2O_2$, respectively (Dahlgren and Karlsson, 1999). In the isoluminol assay, which specifically measures extracellular superoxide, PMNs exposed to AGP after activation demonstrated an ROS production profile intermediate to PMNs exposed to DPI or SOD (Fig. 3D). In the cytochrome c reduction assay, which also specifically measures extracellular superoxide, PMNs exposed to AGP after activation demonstrated a profile of decreased ROS production more similar to PMNs exposed to DPI than to SOD (Fig. 4D). This may suggest that AGP has the potential to exert its effect directly on NADPH oxidase. In contrast, studies measuring extracellular production of hydrogen peroxide revealed that PMNs exposed to AGP prior to activation demonstrate an ROS production profile more similar to PMNs exposed to catalase than to DPI (Fig. 5C). This may suggest that AGP has the ability to act as a scavenger of ROS. Although the exact mechanism of action remains to be elucidated, data presented here suggest that

AGP may directly inhibit NADPH oxidase activity as well as scavenge ROS.

Because it has been previously reported that global inhibition of NADPH oxidase generation of ROS, but not selective scavenging of extracellular ROS, impairs PMN bactericidal activity (Rinaldi et al., 2006), the effects of DPI and AGP on phagocytosis and killing of S. aureus were investigated. DPI, which inhibits NADPH oxidase, had no effect on PMN phagocytosis of S. aureus, but did impair bacterial killing consistent with previous reports (Ellis et al., 1988; Hampton and Winterbourn, 1995; Rinaldi et al., 2006). Exposure of PMN to the highest concentration of AGP that inhibited extracellular superoxide and hydrogen peroxide production, did not affect PMN capacity to phagocytose and kill S. aureus (Table 1). These findings suggest that AGP, through the selective inhibition of extracellular but not global generation of ROS, could have a potential protective role in reducing inflammation-associated tissue damage without impairing the critical role of PMNs to protect the host against invading pathogens.

In the phagocytosis assay, approximately 20% of the PMNs were negative for the presence of intracellular bacteria (Table 1). It is possible that these PMNs may have rapidly killed and degraded phagocytosed bacteria prior to the time of microscopic visualization, and thus, appeared negative for the presence of internalized bacteria. Regardless of the reason why these PMNs did not contain internalized bacteria, it could not be ascribed to a treatment effect as exposure to media or AGP had no effect on either the percentages of PMNs with phagocytosed bacteria or the number of phagocytosed bacteria per PMN.

Through the production of pro-inflammatory cytokines, PMNs play an immunoregulatory role during the course of infection (Peveri et al., 1988; Strieter et al., 1992; Cassatella, 1995). One of the cytokines that activated PMN's produce is IL-8, a pro-inflammatory cytokine that is a potent chemoattractant involved in both the recruitment of PMNs to the site of infection and the stimulation of multiple PMN functions, such as adherence, degranulation, and production of ROS (Peveri et al., 1988). Interestingly, AGP demonstrated the capacity to enhance PMA and zymosan-induced PMN IL-8 production (Fig. 6), and to maximally exert this effect at concentrations (i.e., 400–800 μg/mL) comparable to those present during inflammation (Fig. 6A). Interestingly, a previous study (Lekstrom-Himes et al., 2005) reported that exposure of activated PMNs to ROS inhibitors or scavengers enhances IL-8 production, thus, suggesting a possible inverse relationship between ROS generation and IL-8 production in PMNs. Whether AGP's ability to inhibit PMN ROS generation is mechanistically linked to its ability to enhance IL-8 production remains unknown.

## 5. Conclusions

To our knowledge, this is the first study to demonstrate that the inhibitory effect of AGP on PMN respiratory burst activity is exerted at the level of

extracellular superoxide anion and hydrogen peroxide. Further, this is the first study to investigate the effects of AGP from any species on IL-8 production by any cell type. Although the precise nature of AGP function remains unknown, the findings in this study suggest that AGP may have a divergent effect on PMN inflammatory processes by reducing extracellular ROS release and enhancing IL-8 production. Because of the critical role that PMNs play in the host innate immune response to bacterial infection and the finding that the responses of these cells are moderated by AGP at pathophysiologically relevant concentrations, further studies are warranted to elucidate the specific mechanisms of action by which AGP influences PMN function.

## Acknowledgements

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